PB89-109656

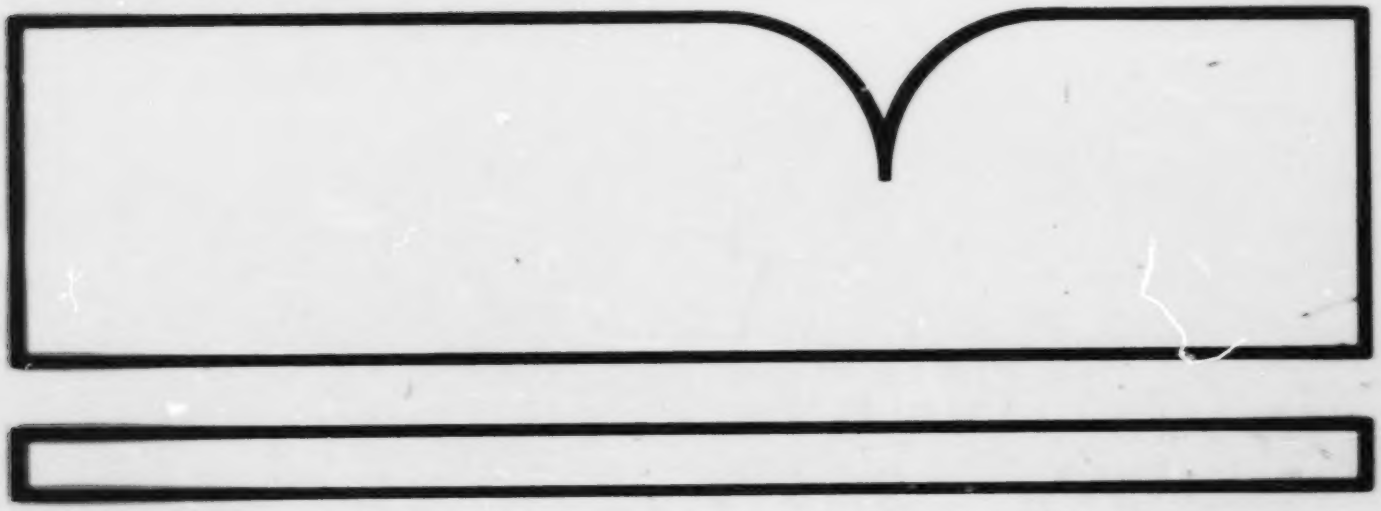
Analysis of Cyclopentafused Isomers of Benz(a)anthracene in Wood Smoke (Journal Version)

North Carolina Univ., Chapel Hill

Prepared for

Health Effects Research Lab.
Research Triangle Park, NC

1987



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1. REPORT NO. EPA/600/J-87/436	2.	3. PB89-109656
4. TITLE AND SUBTITLE Analysis of Cyclopentafused Isomers of Benz(a)anthracene in Wood Smoke (Journal Version)		5. REPORT DATE 1987 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) R.M. Kamens, J.N. Fulcher, L.M. Ball, A. Gold		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Environmental Sciences and Engineering University of North Carolina Chapel Hill, NC 27514		10. PROGRAM ELEMENT NO. 11. CONTRACT/GRANT NO. CR810849 R812256 CR811817
12. SPONSORING AGENCY NAME AND ADDRESS Health Effects Research Laboratory - RTP, NC Office of Research and Development US Environmental Protection Agency Research Triangle Park, NC 27711		13. TYPE OF REPORT AND PERIOD COVERED 14. SPONSORING AGENCY CODE EPA/600/11

15. SUPPLEMENTARY NOTES 1987

Journal Article - Biomedical and Environmental Mass Spectrometry vol. 14, pp. 369-374

16. ABSTRACT

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17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field Group

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NATIONAL TECHNICAL INFORMATION SERVICE
SPRINGFIELD, VA. 22161

18. DISTRIBUTION STATEMENT RELEASE TO PUBLIC	19. SECURITY CLASS (This Report) UNCLASSIFIED	21. NO. OF PAGES 8
	20. SECURITY CLASS (This page) UNCLASSIFIED	22. PRICE

EPA/600/J-87/436
JOURNAL ARTICLE

BIOMEDICAL AND ENVIRONMENTAL MASS SPECTROMETRY, VOL. 14, 369-374 (1987)

Analysis of Cyclopentafused Isomers of Benz(a)anthracene in Wood Smoke

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A previously-unidentified peak occurring in chromatograms of the polycyclic aromatic hydrocarbon fraction from wood smoke particulate extracts analysed by capillary gas chromatography on a DB-5 column was found to correspond in retention index and molecular mass either to benzo(a)fluoranthene or to benzo(l)aceanthrylene, one of a series of four novel and highly mutagenic cyclopentafused isomers of benz(a)anthracene. Detailed sub-fractionation, and re-analysis on a liquid crystal capillary column, indicated that the identity of this peak was most likely to be benzo(a)fluoranthene, a compound not previously reported in woodsmoke.

INTRODUCTION

The recent regain in popularity of wood as a home-heating fuel has raised concern about the potential health implications of exposure to wood smoke. In our laboratory and others, extracts of wood smoke particulate matter have been shown to be mutagenic when assayed in the Ames plate incorporation test with *Salmonella typhimurium* strain TA98 in the presence of exogenous metabolic activation.[1-9] The class of compounds known as polycyclic aromatic hydrocarbons (PAH) are ubiquitous environmental pollutants and can contribute between 10 and 25 percent to the total mutagenicity of wood smoke extracts. In these extracts, this class of compounds is composed largely of unsubstituted (non-alkylated) PAH.[3,8] Many known mutagens such as benzofluoranthenes and benzopyrenes have been identified there,[3,8] but these do not account for the totality of the mutagenicity observed. A series of four cyclopenta-fused PAH, all isomers of benz(a)anthracene (Fig. 1(a-d)), has recently been synthesized[10] and shown to be potent bacterial and mammalian mutagens[11] and in some cases animal carcinogens.[12] One compound of this type, cyclopenta(cd)pyrene, is also a mutagen and has previously been detected in emissions from gasoline-fueled vehicles.[13-16] We were therefore interested in evaluating environmental combustion samples for the presence of these novel compounds, which might be expected to contribute substantially to the overall mutagenic activity.

MATERIALS AND METHODS

Generation of soot samples

Wood smoke emissions were added directly from the chimney of a double door Buck wood stove (Smoky Mountain Enterprises, Asheville, North Carolina USA) to one or both 25 m^3 outdoor Teflon film chambers.[7-9] Split pine logs (6 cm × 10 cm × 50 cm) were used as a fuel and these were burned at a rate of 2 to 6 kg h^{-1}. Initial wood smoke particle concentrations in the chambers ranged from 400 to 2000 $\mu g/m^3$. Soot particles were collected on either 47 mm or 133.4 mm Teflon-impregnated glass fiber filters (Pallflex T60A20). The flow through 47 mm filter samples was 0.07 m^3 min^{-1} and sampling periods generally lasted for 10 min. Particle masses in the range of 0.2 to 1.5 mg were collected. The flow through the 133.4 mm filters was 0.6 m^3 min^{-1}; 10-15 mg of soot particles were collected over a 15 min period.

Extraction procedure

Filter samples were usually extracted within 4 h after collection; when this was not possible, they were held in sealed test tubes at 6°C for no longer than 3 days. The 47 mm filters were extracted with CH_2Cl_2 in 25 ml micro Soxhlet extractors for 16 h. The 133.4 mm filters were extracted with 100 ml CH_2Cl_2 in 140 ml Soxhlet extractors for 16 h. The extract from the micro Soxhlets was concentrated to 3 ml with Supelco micro Snyder columns (Bellefonte, Pennsylvania, USA) and then further concentrated to 50 µl with a dry nitrogen stream. The extract from the 140 ml Soxhlets was rotary evaporated to 5 ml and then concentrated to 100 µl with a dry nitrogen stream.

Isolation of the PAH fraction

To isolate the PAH portion from the raw particulate extract, samples were fractionated with a ternary Spectra Physics 8700 high pressure liquid chromatograph (HPLC; Piscataway, New Jersey, USA) on a 300 × 7.8 mm id Waters semiprep µPorasil column (Milford, Massachusetts, USA) at a flow rate of 2 ml min^{-1}. The solvent was initially 95% hexane in CH_2Cl_2 for 10 min. It was then programmed to 100% CH_2Cl_2 at a rate of 5% per min. After 1 min at 100% CH_2Cl_2, the mobile phase was rapidly ramped up to 100% CH_3CN then

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Abbreviations: PAH, Polycyclic aromatic hydrocarbons; BaA, benz(a)anthracene; BlA, benz(l)aceanthrylene; BaF, benzo(a)fluoranthene; BbF, Benzo(b)fluoranthene; BjF, benzo(j)fluoranthene; BkF, benzo(k)fluoranthene; BaP, benzo(a)fluoranthene; BeP, benzo(e)fluoranthene; HPLC, high pressure liquid chromatograph; GC, gas chromatograph.

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held for 12 min. This is similar to a scheme reported by Alfheim *et al.*[5] and Ramdahl.[17] All solvents used in this study were Burdick & Jackson HPLC grade (American Scientific, Charlotte, North Carolina, USA) unless otherwise stated.

A detailed characterization of this system in our laboratory with injected PAH masses of 25 to 250 ng has shown mean recoveries in excess of 86% with sixteen different PAH, ranging from acenaphthene to benzo(ghi)perylene. More detailed information on the sample work-up procedures and the chambers used in this study have been previously described.[6-9,18]

HPLC subfractionation of the PAH fraction

The Spectra Physics 8700 ternary gradient HPLC pump and programmer were used with a Spectra Physics 8300 fixed wavelength (254 nm) UV detector. Separation was achieved with a 5 μ 4.6 × 150 mm Supelco-PAH analytical reverse phase C18 HPLC column (Supelco Inc., Bellefonte, Pennsylvania, USA). The isocratic mobile phase was 10% H_2O and 90% MeOH, at a flow rate of 0.75 ml min^{-1}. The eluate was collected in approximately 2 ml fractions. Very low PAH recoveries were obtained when we attempted to remove the solvent directly by evaporation under a stream of dry nitrogen gas. It was therefore necessary to solvent exchange into hexane. 2 ml of hexane and 1 ml of H_2O were added to each fraction and the vial containing the fraction was shaken vigorously. The hexane layer was drawn off with a 2 ml syringe and the extraction procedure was then repeated 3 more times with the remaining MeOH and H_2O. This produced fractions which were now in approximately 10 ml of hexane. After concentration of this volume to 50 μl with a dry nitrogen stream, the PAH recovery for the entire solvent exchange procedure was approximately 30% to 40%.

Analysis by gas chromatography

Analysis on DB-5 fused silica columns. A fifteen or thirty meter 0.25 mm i.d. J & W DB-5 fused silica column (Alltech Associates, Deerfield, Illinois, USA) was used in either a Carlo Erba model 4130 gas chromatograph or a Perkin Elmer 900 gas chromatograph (modified for capillary use) with flame ionization detection. Hydrogen was used as a carrier with 10 psi head pressure on the column. One microliter samples were injected into a 300°C split/splitless injector while the oven was held at 40°C. After 20 s, the split valve was opened and the oven quickly ramped to 130°C. The oven was then programmed at either 6 or 8°C to 290°C and held for 8 min. Retention indices for each of the cyclopenta-PAH compounds were estimated[19] by predicting the elution time of benzo(b)fluoranthene (BbF) and benzo(a)pyrene (BaP) with respect to the internal standard, β,β-binaphthyl. These were then used as bracket references.

Analysis on a bonded liquid crystal column. Liquid-crystal-fused-silica-columns provide either better resolution or a different elution order for selected PAH than the bonded or nonbonded phase methylsilicone columns (J & W DB-5, DB-1, Supelco SP21000, SE-54, SE-30). The capillary liquid crystal column used on this project was a 30 m fused silica MPMS (0.25 mm i.d.) column purchased from Alltech Associates (Deerfield, Illinois, USA). The disadvantages of these columns are that they require gentle 'break-in' conditions, can be easily deactivated, and cannot tolerate injections containing chlorinated solvents. This necessitates that samples be solvent exchanged into hexane, benzene or MeOH.

Injection was at 40 °C into a split-splitless injector at 280 °C. After 20 s the GC oven was quickly heated to 180 °C and programmed at 6 °C to 265 °C; Helium was used as the carrier gas with 20 psi head pressure on a Perkin-Elmer 900 gas chromatograph modified for capillary use.

Mass spectrometry analysis

The PAH fraction was analysed on a VG-Micromass 7070F (Vacuum Generators, Altrincham, Cheshire, UK) double-focusing magnetic sector instrument interfaced with a Hewlett-Packard 5710A (Hewlett-Packard Co., Palo Alto, California, USA) gas chromatograph fitted with a DB-5 30 m fused silica capillary column. Injections (1 μl, CH_2Cl_2) were made on-column (SGE OCI-II injector) at room temperature. The oven temperature, originally at 40 °C, was ramped to 100 °C, then temperature-programmed from 100 to 290 °C at 6 °C min^{-1}. The mass spectrometer was operated in the electron ionization mode, at source pressure 5×10^{-6} torr, temperature 220 °C, electron energy 70 eV, current 200 μA. The output was scanned at 0.7 sec $decade^{-1}$, at a resolution of 1000 (10% valley). Data were recorded, stored and processed on a VG 2035 Data System.

Chemicals

The cyclopentafused isomers of benz(a)anthracene (Fig. 1) were available from previous synthesis.[10] Benzo(b) and (k)fluoranthene isomers were purchased from Supelco Inc (Bellefonte, Pennsylvania; PAH standards #4-9155), and a standard PAH mixture (SRM #1647) was obtained from the National Bureau of Standards (Gaithersburg, Maryland, USA). Other standard compounds were obtained from commercial sources, generally Aldrich Chemical Co. (Milwaukee, Wisconsin, USA).

RESULTS AND DISCUSSION

Retention indices (shown in Table 1) were estimated for the four cyclopentafused PAH (Fig. 1) in order to evaluate where to search for these compounds in a standard PAH analysis scheme. The retention indices indicated that they may be expected to elute on a DB-5 capillary column in the same region as the benzofluoranthenes and benzopyrenes. Analysis of the PAH fraction of a wood smoke sample (WIB collected 4/10/85) by GC/MS (Fig. 2) showed a cluster of unidentified peaks with molecular mass 252 (corresponding to the molecular weight of our cyclopentafused PAH) between benzo(k)fluoranthene (BkF; Scan #1611) and benzo(e)pyrene (BeP; Scan #1657). In particular, one

Table 1. Chromatography of cyclopentafused isomers of benz(a)anthracene and related compounds

Compound	Relative Retention time[a]	Retention Indices[b]
Benzo(a)pyrene	1.11250	453.44
Benz(k)acephenanthrylene	1.08803 ± 0.00174	447.62
Benz(e)aceanthrylene	1.08785 ± 0.00058	447.58
Benz(j)aceanthrylene	1.08640 ± 0.00041	447.17
Benz(l)aceanthrylene	1.08074 ± 0.00031	445.68
Benzo(b)fluoranthene	1.06476	441.74
Peak #20a	1.08132 ± 0.00058	445.5–445.85

[a] Relative retention times (±SD, $n=6$) compared to the internal standard β, β-binaphthyl (24.48 ± 0.04 min.) on a 15 m DB-5 fused silica capillary column with H_2 carrier, oven at 40 °C, ramped immediately to 100 °C, then temperature-programmed to 300 °C at 4 °C/min^{-1}.

[b] Retention indices estimated[19] by computing the retention times of BbF and BaP in similar samples with respect to β, β-binaphthyl and using these values as bracket references for the other compounds.

large peak in the PAH fraction was detectable, at scan #1628, which was designated #20a because it ran between peak #20, BkF, and peak #21, BeP, in our previously published chromatography scheme.[8] Fig. 3 illustrates a representative FID chromatogram of this PAH fraction. A similar unidentified peak was observed by Ramdahl and co-workers[3] eluting between BbF (peak #21 here) and BeP (peak #22). (The isomers benzo(b), (j) and (k)fluoranthene all generally co-elute on this type of capillary GC column). Peak #20a was tentatively identified as benzo(l)aceanthrylene (BlA) based on its molecular mass and retention index (Table 1). However this tentative identification was cast into doubt when a sample of the previously unavailable isomer benzo(a)fluoranthene (BaF) was obtained from Dr S. Wise (U.S. National Bureau of Standards). Its calculated retention index (445.19) suggested that BAF would be hard to separate from BlA on a DB-5 column, and this proved to be the case. The PAH fraction was therefore subfractionated on a reverse phase HPLC system in which BaF and BlA showed baseline resolution. This produced two subfractions of interest which were

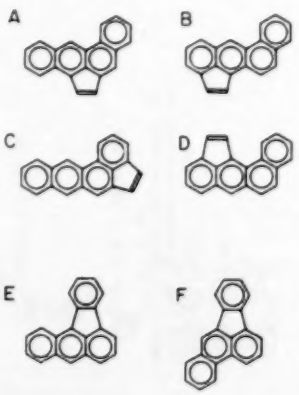

Figure 1. Structures of cyclopentafused isomers of benz(a)anthracene and related compounds. (A) benz(e)aceanthrylene, (B) benz(j)aceanthrylene, (C) benz(k)acephenanthrylene, (D) benz(l)aceanthrylene, (E) benzo(a)fluoranthene, (F) benzo(b)fluoranthene.

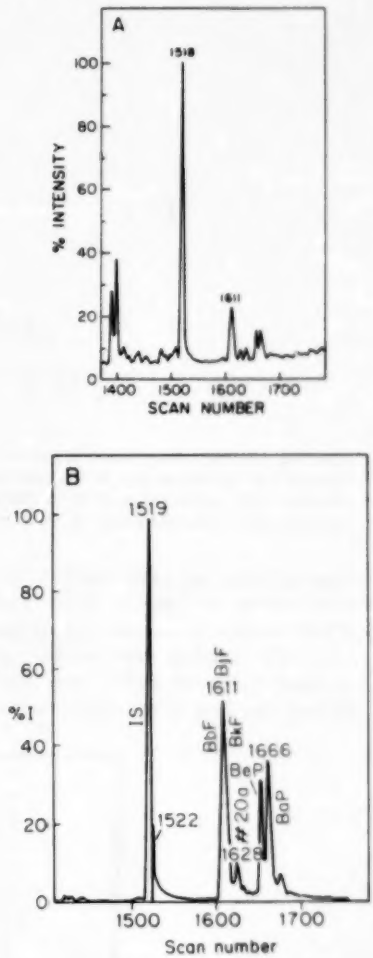

Figure 2. Reconstructed ion chromatogram of the polycyclic aromatic hydrocarbon fraction from a wood smoke particulate extract, showing: A, total ion current; B, ion current m/z 252 (10 scans/11 sec). IS, internal standard, β, β-binaphthyl, scan #1519; benzo(b), (j) and (k)fluoranthenes, scan #1611; benzo(e)pyrene, scan #1657; benzo(a)pyrene, scan #1666.

labeled 'A' and 'B'. According to the HPLC fractionation scheme, fraction 'A' should contain lighter molecular weight compounds like fluoranthene, pyrene, benz(a)anthracene, and chrysene as well as BaF. Fraction 'B' should contain BbF, BkF, BlA, BeP and BaP.

GC analysis of subfractions A and B from three separate wood smoke samples (collected 1/21/85) on the DB-5 column gave inconclusive results, for reasons including poor mass recovery and possible sample contamination. The peak areas for benzo(a)anthracene, chrysene and triphenylene suggest that only 30% of these compounds were recovered in subfraction A.

The 'A' and 'B' fractions were also concentrated to 10 µl and further analyzed on a VG 7070F Micromass GC/MS system. An on-column injection apparatus was used along with the same column and temperature program previously described in this section. Analysis of the 'B' fraction did not show any m/e 252 ions in the BlA region.

Another sample was collected from the chambers in September 1985. Approximately 125 mg of wood soot

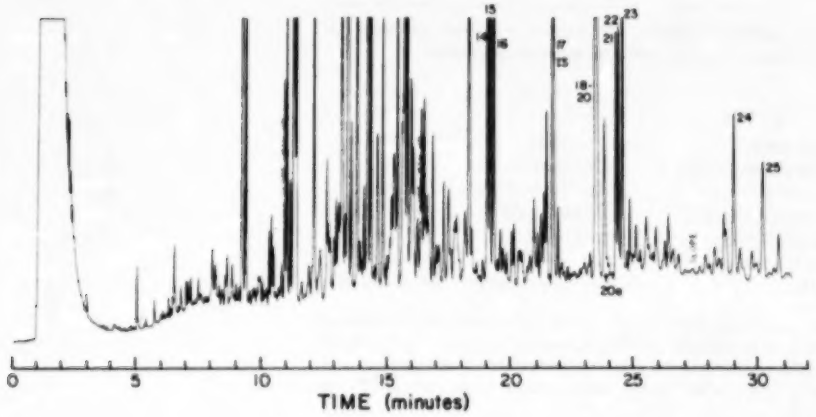

Figure 3. Capillary gas chromatogram of unfractionated PAH extract from 4-10-85, sample WIB, on a 30 m DB-5 fused silica column, with hydrogen as the carrier gas. IS, internal standard, β, β-binaphthyl; peak identities, #14, cyclopenta(cd)pyrene, #15, benz(a)anthracene, #16, chrysene and triphenylene, #18–20, benzo(b), (j) and (k)fluoranthenes, #20a, unknown, #21, benzo(e)pyrene, #22, benzo(a)pyrene, #23, perylene, #24, indeno(cd)pyrene, #25, benzo(ghi)perylene.

was collected and extracted in a 140 ml Soxhlet extractor with 100 ml of CH_2Cl_2. After separation of the entire PAH fraction from the rest of the particulate extract, the PAH fraction was subfractionated by the HPLC method discussed above. Very 'tight' fractions were collected: the first in the region where BaF was expected to elute, and the second in the region where BIA was expected to elute.

Four aliquots of the September PAH fraction were thus subfractionated (a total of ~10 μg), and the individual subfractions were pooled, solvent exchanged into hexane, concentrated and re-analysed on a DB-5

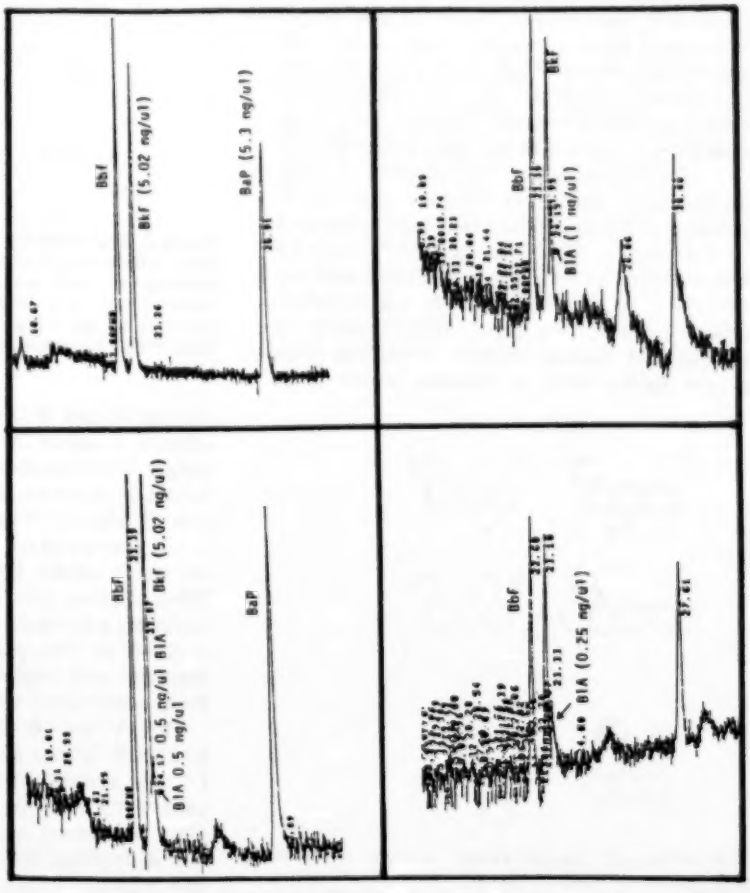

Figure 4. Standards of benzo(k)fluoranthene (5.02 ng μl^{-1}) and benzo(l)aceanthrylene (0–1 ng μl^{-1}) spiked into a standard mix of PAH (NBS SRM #1647), analysed by gas chromatography, on a liquid crystal capillary column with helium as the carrier gas as described in the text.

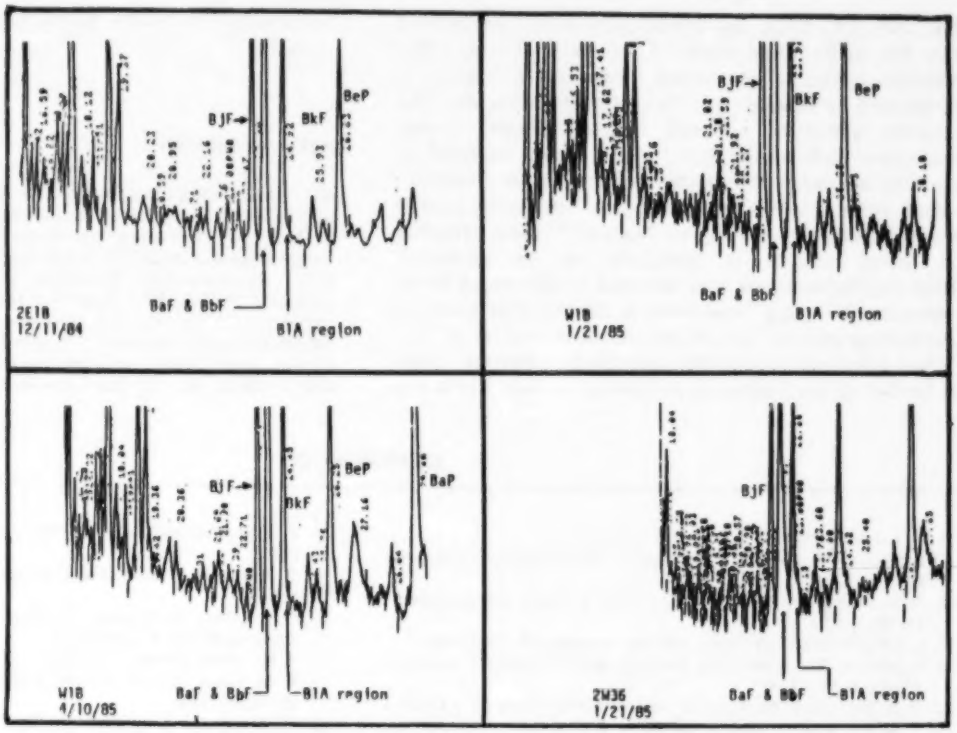

Figure 5. Unfractionated PAH fractions from wood smoke analysed on a 30 m × 0.25 mm PMPS bonded fused silica liquid crystal capillary column.

column. The chromatograms from the first subfraction (fraction A) had a sizeable peak in the BaF/BIA region, indicating that peak #20a was composed primarily of BaF. The peak in the second subfraction (fraction B) eluted with a retention time less than that of BIA, and could be either BbF, BjF or BkF. We have therefore concluded that if BIA was present in this sample it is there at only trace amounts and at concentrations which were below the detection limit of our analytical procedures.

Analysis by liquid crystal capillary gas chromatography

As an alternative approach, we investigated the use of a liquid crystal fused silica capillary column which was expected to provide a different elution order for PAH. With hydrogen as the carrier gas, excellent separation of benz(a)anthracene (BaA) and chrysene can be achieved. Chrysene and triphenylene, which co-elute on a DB-5 column can also be resolved. Benzo(j)fluoranthene (BjF) and BkF are separated, but BbF and BaF co-elute. BIA and BeP eluted very close to one another but were resolvable. The major problem that we encountered was that BIA was apparently hydrogenated by the reducing conditions of the H_2 carrier and we could not obtain good sensitivity for this compound.

The use of helium remedied this difficulty at the expense of slight peak broadening. BjF eluted the earliest of the compounds of interest; BaF and BbF co-eluted next, well separated from BkF. The retention of BIA, however, was shifted so that it emerged from the column just after BkF and was not completely resolved from BkF. Since we suspected that BkF would be present in much higher concentrations than BIA, we made up a series of standards with both BkF and BIA. BkF was at a concentration of 5 ng μl^{-1} and BIA was varied from 1 to 0.25 ng μl^{-1}. As can be seen in Fig. 4, one ng μl^{-1} of BIA in the presence of 5 ng μl^{-1} of BkF can easily be seen; as we approach 0.5 and 0.25 ng μl^{-1} the BIA peak becomes more and more fused with the high concentration BkF peak. We feel that the minimum detectable concentration for BIA is in the range of 0.2 ng μl^{-1}; concentrations in the 0.1 ng ml^{-1} range would have the appearance of an unresolved shoulder on the BkF peak. With this column, however, pure BkF at 5 ng μl^{-1} has a reasonably sharp peak (Fig. 4) and one may speculate that 'tailing' of the BkF peak in an environmental sample may possibly be due to trace amounts of BIA. Inspection of the chromatograms in Fig. 5 from four different unfractionated wood soot PAH samples (2E1B, collected on 12-11-84, W1B and W3B from 1-21-85, and W1B from 4-10-85) did no more than suggest a shoulder on the trailing side of the BkF peak. Even the W1B unfractionated PAH sample from 4-10-85, which had a very strong peak #20a in the BIA/BaF region of the DB-5 capillary GC chromatogram (Fig. 3), does not show any distinguishable shoulder on the BkF peak. As BaF and BbF co-elute on this column, and BbF is a common environmental contaminant, the presence of a strong peak in the BaF/BbF region does not in itself signify the presence of BaF in these samples.

Therefore by two separate chromatographic methods, at most trace amounts of BIA can be detected in wood smoke extracts, and none of the other three cyclopentafused isomers of benz(a)anthracene. Based on

GC/MS, GC/FID, and HPLC separation, we believe that the unidentified peak (#20a) detectable in DB-5 chromatograms of the wood smoke PAH fraction is composed primarily of benzo(a)fluoranthene. The isomers benzo(b), (j) and (k)fluoranthene, known mutagens and carcinogens,[20] have been reported in ambient air particulate matter collected from American urban areas (17-49 $\mu g\, g^{-1}$ particles)[21] and from a small woodburning community in Norway;[22] their presence in wood smoke is therefore to be expected. Benzo(a)fluoranthene was reported in very much lower amounts (0.21 $\mu g\, g^{-1}$ particles) in the urban ambient air particulate matter,[21] and in an extract of coal tar pitch.[23] It has not been specifically identified elsewhere. Little is known of the biological properties of BaF, but it can be anticipated to be genotoxically active as are its isomers.

Acknowledgments

We thank Ms Jean Perry for technical assistance, Ms D. E. Plummer and Ms F. L. Dancy for typing this manuscript, and Dr S. Wise of the U.S. National Bureau of Standards, Gaithersburg, Maryland, USA, for the sample of benzo(a)fluoranthene. This work was supported by U.S. Environmental Protection Agency (EPA) Contract #P05D2775NTEX, EPA Grant R #812256, and EPA Co-operative Agreement CR #811817. This article has been subjected to the Health Effects Research Laboratory, US EPA's peer review, and approved for publication. It does not necessarily reflect the views of the Agency, and no official endorsement of any kind should be inferred.

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